1-1-2004

Plasminogen binding by group A streptococcal isolates from a tropical region with hyperendemic streptococcal skin infection and a high incidence of invasive infection

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Abstract

Reports of resurgence in invasive group A streptococcal (GAS) infections come mainly from affluent populations with infrequent exposure to GAS. In the tropical Northern Territory (NT) of Australia, high incidence of invasive GAS disease is secondary to endemic skin infection; serotype M1 clones are rare in invasive infection; the diversity and level of exposure to GAS strains is high and no particular strains dominate. Expression of a plasminogen-binding group A streptococcal M-like protein (PAM) has been associated with skin infection in isolates elsewhere (Bessen, D., C.M. Sotir, T.M. Readdy, and S.K. Hollingshead.1996. J. Infect. Dis. 173:896-900) and subversion of the host plasminogen system by GAS is thought to contribute to invasion in animal models. Here, we describe the relationship between plasminogen-binding capacity of GAS isolates, PAM genotype and invasive capacity in 29 GAS isolates belonging to 25 distinct strains from the NT. In the presence of fibrinogen and streptokinase, invasive isolates bound more plasminogen than isolates from uncomplicated infections ($p \leq 0.004$). Only PAM-positive isolates bound substantial levels of plasminogen by a fibrinogen-streptokinase-independent pathway (direct binding). Despite considerable amino acid sequence variation within the A1 repeat region of PAM where the plasminogen-binding domain maps, the critical lysine residue was conserved.

Keywords

plasminogen, streptococcus, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

This article was originally published as McKay, FC, Plasminogen binding by group A streptococcal isolates from a tropical region with hyperendemic streptococcal skin infection and a high incidence of invasive infection, Infection and Immunity, 72(1), 2004, 364-370.

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Plasminogen binding by group A streptococcal isolates from a tropical region with hyperendemic streptococcal skin infection and a high incidence of invasive infection

Running Title: Plasminogen binding by Northern Territory S. pyogenes

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Part of this work was presented as a scientific poster at the XVth Lancefield International Symposium on Streptococci and Streptococcal Diseases, October 6-11, 2002, Goa, India (abstract P2.26).

Abstract

Reports of resurgence in invasive group A streptococcal (GAS) infections come mainly from affluent populations with infrequent exposure to GAS. In the tropical Northern Territory (NT) of Australia, high incidence of invasive GAS disease is secondary to endemic skin infection; serotype M1 clones are rare in invasive infection; the diversity and level of exposure to GAS strains is high and no particular strains dominate. Expression of a plasminogen-binding group A streptococcal M-like protein (PAM) has been associated with skin infection in isolates elsewhere (Bessen, D., C.M. Sotir, T.M. Readdy, and S.K. Hollingshead.1996. J. Infect. Dis. 173:896-900) and subversion of the host plasminogen system by GAS is thought to contribute to invasion in animal models. Here, we describe the relationship between plasminogen-binding capacity of GAS isolates, PAM genotype and invasive capacity in 29 GAS isolates belonging to **25** distinct strains from the NT. In the presence of fibrinogen and streptokinase, invasive isolates bound more plasminogen than isolates from uncomplicated infections ($p \leq 0.004$). Only PAM-positive isolates bound substantial levels of plasminogen by a fibrinogen-streptokinase-independent pathway (direct binding). Despite considerable amino acid sequence variation within the A1 repeat region of PAM where the plasminogen-binding domain maps, the critical lysine residue was conserved.

Introduction

GAS cause a variety of superficial infections such as impetigo, as well as invasive diseases including bacteremia, necrotizing fasciitis and myositis. A recent survey from the tropical "Top End" of the NT found a high incidence of invasive GAS disease, with rates of bacteremia in non-Aboriginal people (9) comparable to those constituting the resurgence in GAS bacteremia described elsewhere in the Western World (30, 40). Amongst Aboriginal people of the Top End the incidence of GAS bacteremia is 5 times that of non-Aboriginal people living in the same region (9).

In addition to the high incidence of severe GAS infection in the NT, several other epidemiological features characterise the GAS isolates from this vast geographic region. Up to 60% of isolates from the NT do not react with available M-typing sera (29), while some react with more than one, giving an ambiguous M-typing result (28). This has lead to the development of the *vir* typing system, consisting of restriction fragment length polymorphism analysis of the *mga* regulon (15). *Vir*-typing of strains causing GAS infection in Aboriginal communities has demonstrated that the diversity and turnover rate of these strains is much higher than those reported elsewhere (8). There has been no evidence of a resurgence in severe invasive infections due to particular strains or any invasive infection associated with M1 clones (9) which have been responsible for much of the recent resurgence in GAS bacteremia reported elsewhere (32). A recent NT study examining 100 GAS isolates found only 2 M1 isolates, which were not clonally related or isolated from invasive disease cases (R. J. Towers, G. Mollinari, K. Bruder-Okando, D. Zell, A. Delvecchio, P. Fagan, S. Gardam, M. Hibble, B. Currie, M. J. Walker, K. S. Sriprakash, and G. S. Chhatwal, Abstr. XV Lancefield International Symposium on

Streptococci and Streptococcal Diseases, abstr. P1.02, 2002) Similarly, a study of invasive GAS disease from 1996-2001 in Townsville Hospital, North Queensland, found only 5.5% were emm1 (gene for M1 protein) sequence type (R. Norton, H. V. Smith, N. Wood, E. Siegbrecht, A. Ross, and N. Ketheesan, Abstr. XV Lancefield International Symposium on Streptococci and Streptococcal Diseases, abstr. O2.2, 2002)

The most common focus of invasive infection in the NT is the skin, with no episodes of pharyngitis underlying GAS bacteremia in a 6-year retrospective review (9). Rates of GAS skin infection are extremely high, due in part to infection of scabies lesions, with impetigo prevalence rates of up to 70% reported (17).

An important role for plasminogen in invasion of host tissue barriers has been recognised in several bacterial species (6, 12, 24). Plasminogen is the zymogen of the broad-spectrum serine protease plasmin, which degrades fibrin clots and extracellular matrix (ECM) components such as fibronectin, laminin, vitronectin, and proteoglycans. It also activates matrix prometalloproteases -1, -3, -9 and -14, which cleave other components of the ECM such as collagen (41). Plasminogen is activated to plasmin by the host activators tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). Co-localisation of plasminogen and tPA to fibrin(ogen) results in fibrinolysis, while uPA is the major cell-bound activator, functioning in cellular invasion (23). Bacterial plasminogen activators include Pla of *Yersinia pestis* and streptokinase of group A, C and G streptococci (6).

Plasminogen binds to the high affinity GAS plasminogen receptors plasminogen-binding group A streptococcal M-like protein (PAM) (3) and streptococcal enolase (SEN) (26) by its amino-terminal lysine-binding kringle domains (22). Cell-surface

glyceraldehyde-3-phosphate dehydrogenase, designated Plr (42) or streptococcal dehydrogenase (SDH) (27), has been reported to bind plasminogen with low affinity.

Plasminogen binds to PAM via lysine residues present in two tandem repeats, A1 and A2, in the amino-terminal variable region of PAM (3). The lysine residue found in A1 is responsible for the majority of the plasminogen binding capacity of PAM (44). This interaction is at least partly mediated by kringle 2 of plasminogen (43).

The PAM phenotype and genotype have been found to be selectively distributed among GAS strains of *emm* pattern D (33), reported to be a genetic marker for skin as the preferred tissue site for infection (4), (5). It has been suggested that plasminogen binding capacity of PAM may be important for persistence of GAS in skin infection. Proteolytic capacity of PAM-bound plasminogen on the surface of GAS may allow the breakdown of fibrin barriers formed during wound healing. Alternatively, sequestering of plasminogen by GAS may diminish the plasminogen available for host immune response at the infection site (42).

An indirect plasminogen-binding pathway in GAS with a requirement for streptokinase and fibrinogen has also been described (38, 39). Plasminogen binding by this pathway has been correlated with the fibrinogen-binding capacity of various GAS strains (39), and it has been suggested that fibrinogen bound to M or M-related proteins mediates capture of fibrinogen-streptokinase-plasminogen complexes to the GAS cell surface (11, 38). Evidence for the formation of the trimolecular complex using purified protein components has been reported previously (34). The trimolecular complex bound to the GAS cell surface possess both plasmin (38) and plasminogen-activator (13) activities.

In a mouse skin infection model, GAS preincubated with human plasminogen, human fibrinogen and exogenous streptokinase were more invasive than untreated bacteria (25). However, a study of clinical isolates found no significant association between plasminogen binding and the invasive potential of the isolates, although the mean plasmin activity of blood isolates was higher than that of throat isolates (37). To date, there has been no definitive epidemiological evidence supporting a relationship between the human plasminogen system and GAS in accentuating virulence (6).

Reports suggesting an important role for plasminogen in both GAS skin disease and invasive disease led us to investigate aspects of plasminogen binding by GAS isolates from the NT where a high incidence of both clinical manifestations of GAS infection prevails. Isolates from uncomplicated infections and invasive disease cases were examined for plasminogen binding by the direct and fibrinogen-streptokinase-dependent (FSD) pathways, fibrinogen binding capacity, PAM genotype, *emm*-pattern and *emm* sequence type.

Materials and Methods

Proteins. Glu-plasminogen was purified from human plasma as previously described (1). Human fibrinogen (essentially plasminogen-free; Sigma, St Louis, MO) was tested to confirm the absence of contaminating fibronectin by Western blotting with a rabbit polyclonal antibody against human fibronectin (Sigma, St Louis, MO). Plasminogen and fibrinogen were labelled with ^{125}I (Amersham Biosciences, Arlington Heights, IL) by the chloramine T method (20). All labelled proteins were analysed for homogeneity by gel electrophoresis and autoradiography in comparison with non-labelled

proteins.

Bacterial strains, media and growth conditions. Group A streptococcal isolates were collected from patients in the Northern Territory between 1990 and 1998. Isolates were collected from either invasive infections (from normally sterile tissues such as blood) or uncomplicated infections (from non-sterile tissues such as skin). The isolates were largely from Aboriginal Australians due to high endemicity of GAS disease in this population (9). GAS isolates were cultured overnight at 37ºC in Todd-Hewitt broth (THB; Difco, Detroit, MI) supplemented with 1% yeast extract. Cells were washed twice with 5ml of PBS (0.01M phosphate, 0.14M sodium chloride, pH 7.4) containing 0.05% Tween 20 (PBST) and resuspended to an optical density (OD) of 1.0 at 600nm in PBST with 100mM of the lysine analogue epsilon-amino caproic acid (eACA; Sigma, St Louis, MO) added where appropriate.

Molecular typing and emm pattern. *Vir* typing (15) was performed as previously described. The method used to determine *emm* sequence types was modified from the *Streptococcus pyogenes emm* sequence database (2) and is described briefly here. Chromosomal DNA was prepared using the DNeasy Tissue Extraction kit (Qiagen, Hilden, Germany). PCR-amplification of chromosomal DNA was performed using primers emm1 and emm2 (2) or using primers VUF and SBR as previously described (16). The resulting PCR product was purified using a MinElute Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced with BigDye™ Ready Reaction Mix (Applied Biosystems, Boston, MA) using emm 1 or emmseq2 primers. The resulting sequences were compared to *emm* sequences in the *Streptococcus pyogenes emm* sequence database (2).

Determination of *emm* pattern was performed as previously described (4, 18) except that annealing temperatures were varied between 56ºC and 62ºC.

Binding assays. The method used to measure the binding of radiolabelled proteins was essentially as previously described (10). Labelled protein (approximately 70ng of ^{125}I-plasminogen, or 45ng of ^{125}I-fibrinogen) was added to 250µl of the cell suspension and incubated for 45 min at room temperature. When required fibrinogen (1.5-fold molar excess compared to plasminogen) and streptokinase (10-fold molar excess compared to plasminogen; Sigma, St Louis, MO) were added to the cell suspension. Equimolar plasminogen and streptokinase were added to the cell suspension for selected assays. The cells were sedimented by centrifugation and the supernatant carefully aspirated. Pellet-associated radioactivity was measured using an automatic gamma counter (Wallac) and the results were expressed as a percentage of input radioactivity. All measurements were determined in triplicate.

DNA methods. Chromosomal DNA was isolated from streptococcal isolates using the Instagene method (Bio-Rad Laboratories, Hercules, CA). Genes encoding M and M-like proteins from GAS strains were PCR-amplified from chromosomal DNA using primers M1 and M2 (3)**.** The SEN gene was PCR-amplified from chromosomal DNA using SENF1 and SENR1 (Table 1). PCR cycling temperatures were 94ºC, 50ºC and 72ºC for denaturation, annealing and extension reactions respectively. Thirty and thirty-two cycles were used for amplification of genes encoding M and SEN, respectively.

For Southern hybridisation (31) of PAM genes, PCR products amplified using the M1 and M2 primers were resolved by agarose gel electrophoresis, transferred to a

Hybond N+ Nylon membrane (Amersham Biosciences, Arlington Heights, IL) and cross-linked to the membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Prehybridisation, hybridisation and detection were performed using a digoxigenin-11-ddUTP labelling kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions, except that blocking was performed with 5% skim milk powder in maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5). The PAM oligonucleotide probe (5'-CGACTTAAAA(A/G)CGAGAGACATGA-3') is homologous to the sequence corresponding to the most conserved residues (RLKN/SERH) in the A1 (DAELQRLKNERHE) and A2 (EAELERLKSERHD) repeats of PAM. The probe was 3'-end labelled with digoxygenin-11-ddUTP according to the manufacturer's instructions.

PCR products amplified using the M1 and M2 primers from PAM-positive GAS strains were cloned into plasmid pCR®2.1 using the TA Cloning Kit (Invitrogen, Carlsbad, CA). *Escherichia coli* INVαF' (Invitrogen, Carlsbad, CA) were transformed with plasmids containing the PAM genes according to the manufacturer's instructions. Plasmid DNA was extracted using a QIAprep Spin Miniprep kit (Qiagen, Hilden, Germany). Plasmids were screened for the presence of the cloned PAM gene by Southern hybridisation.

DNA sequences were determined using Terminator Ready reaction mix (PE Applied Biosystems, Boston, MA) and appropriate primers (Table 1).

Statistical analysis. Pearson's χ^2 analysis was used to determine associations between genetic markers and invasive classification. Binding data were analysed for differences between PAM status and infection classification using two-way analysis of variance (ANOVA). Where data (or arcsine-transformed data) did not fit the assumptions

for two-way ANOVA, an unpaired Student's *t* test, Welch ANOVA (unequal variances) or Wilcoxan/Kruskal-Wallis Rank Sum test (non-parametric) was used to compare groups separately. A paired Student's *t* test was used to analyse responses of isolates to different experimental conditions. Pearson's (parametric) or Spearman's (non-parametric) correlation coefficients (*r*) were used to describe the relationships between cell-surface binding of radiolabelled proteins.

Amino acid sequence accession numbers. The DNA sequences of the A1 and A2 repeat regions of PAM genes were deposited in Genbank. The corresponding accession numbers for each of the PAM-positive isolates is given in parentheses: NS10 (AY351846); NS1133 (AY351847); NS59 (AY351848); NS50.1 (AY351849); NS32 (AY351850); NS13 (AY351851); NS53 (AY351852); NS253 (AY351853); NS223 (AY351854); NS265 (AY351855); NS88.2 (AY351856); NS455 (AY351857); and NS696 (AY351858).

Results

Genetic analysis of GAS isolates. Characteristics of the 29 clinical isolates from invasive cases (n = 14) and uncomplicated (n = 15) GAS infections are given in Table 2. The isolates showed considerable genetic diversity, with 25 *vir* types, 24 e*mm* sequence types and 4 of the five major *emm* patterns represented (Table 2). Together the 29 isolates represent 25 genetically distinct strains which are thus not epidemiologically linked. There were 3 small clusters of isolates which were found to be genetically indistinguishable based on the typing methods used. These were NS730 and NS733 (*vir* type 2.2, *emm*ST 90 and *emm* pattern E); NS10, NS13 and NS59 (*vir* type 24, *emm*ST 53

and *emm* pattern D); and NS455 and NS253 (*vir* type 29.1, *emm*ST 52 and *emm* pattern D). In this study *emm* pattern was not significantly associated with invasive disease ($p \leq 0.148$).

GAS isolates were then examined for presence of genes encoding the high affinity plasminogen binding receptors, SEN and PAM, and for genetic variation within the plasminogen binding domains of these receptors. All the isolates contained the SEN gene with both plasminogen binding C-terminal lysine residues (14, 26) conserved (data not shown). Of the 14 isolates from invasive disease cases, 5 were PAM-positive by Southern hybridisation analysis, while 8 of the 15 isolates from uncomplicated infections were PAM-positive.

The A1 and A2 repeats of the PAM sequences demonstrate divergence compared to the repeats in the prototype PAM (3) (Figure 1). Isolates of the same *vir* type had identical A1 and A2 repeats. Except for NS696, all the 13 PAM-positive isolates possessed *emm* pattern D *mga* regulon.

Plasminogen binding by GAS isolates. To investigate whether plasminogen binding is associated with the invasive phenotype in NT isolates, isolates from invasive disease and uncomplicated infections were compared for this property. Binding by the direct and FSD pathways was measured (Figure 2). In the presence of eACA, a competitive inhibitor of lysine-dependent binding, binding was reduced to 4% of input plasminogen (range 2-11%), except in isolate NS 88.2, which showed high level of lysine independent binding of plasminogen (17%; data not shown).

Presence of the PAM gene had a significant positive effect on plasminogen binding by both the direct ($p \leq 0.036$) and FSD ($p \leq 0.026$) pathways. Invasive isolates

bound more plasminogen directly than isolates from uncomplicated infections in the PAM-positive group ($p \leq 0.027$). In the PAM-negative group all of the isolates displayed minimal reactivity with plasminogen (7% mean) and there was no difference in direct plasminogen binding capacity between invasive and non-invasive isolates ($p \leq 0.180$).

In the presence of streptokinase and fibrinogen, isolates from invasive disease cases bound more plasminogen than isolates from uncomplicated infections ($p \leq 0.004$ for effect of invasive phenotype in 2-way ANOVA; $p \leq 0.026$ for effect of PAM genotype). There was an absolute requirement for both fibrinogen and streptokinase to significantly increase plasminogen binding in a representative isolate (Figure 3). The enhancement of plasminogen binding did not occur when streptokinase alone or fibrinogen alone was added ($p \geq 0.05$ for both conditions).

Within the small clusters of genetically indistinguishable isolates identified, invasive isolates bound more plasminogen than isolates from uncomplicated infection by both the direct and FSD pathways in each case.

Relationships between fibrinogen and plasminogen binding. The relationship between fibrinogen-binding and FSD plasminogen binding by the NT GAS isolates was investigated to determine whether the data is consistent with a model of capture of fibrinogen-streptokinase-plasminogen complexes by fibrinogen binding proteins (37). Binding of fibrinogen to GAS isolates is shown in Figure 4A. PAM-positive isolates bound more fibrinogen than PAM-negative isolates ($p \leq 0.019$). There was a correlation between fibrinogen binding and FSD plasminogen binding among PAM-positive isolates ($r = 0.775$, $p \leq 0.002$; Figure 4A). By contrast PAM-negative isolates do not show such correlation ($r = 0.035$, $p \leq 0.897$). Nor was there a correlation between fibrinogen binding

and the increase in plasminogen binding resulting from addition of streptokinase and fibrinogen ($r = 0.007$, $p \leq 0.778$)

In PAM-positive isolates there was a strong correlation between direct plasminogen binding and FSD plasminogen binding ($r = 0.902$, $p \leq 0.0001$; Figure 4B), and between direct plasminogen binding and fibrinogen binding ($r = 0.806$, $p \leq 0.001$).

Discussion

Subversion of the host plasminogen system renders a pathogen capable of degrading ECM proteins and activating a cascade of metalloproteases, conferring the potential to invade host tissue barriers. An important role for plasminogen in the invasive process of GAS has been demonstrated in an animal model (25), but as yet no definitive epidemiological evidence has supported the hypothesis that the human plasminogen system plays a role in GAS invasive disease.

To our knowledge this is the first report to demonstrate a significant relationship between acquisition of plasminogen by human clinical isolates from a range of *emm* sequence types of GAS and propensity to cause invasive diseases. Amongst PAM-positive isolates which are capable of binding plasminogen directly, invasive isolates bind more plasminogen both directly, and by a FSD pathway which is correlated with direct plasminogen binding. Within the small clusters of PAM-positive isolates indistinguishable by the genotyping methods used, invasive isolates always bound more plasminogen than isolates from uncomplicated infection by both the direct and FSD pathways. Possible reasons for plasminogen binding differences in these clusters might include variation in expression levels or differential post-translational processing or

degradation. For PAM-negative isolates which do not bind substantial amounts of plasminogen directly, invasive isolates bind more plasminogen by the FSD pathway. Together these results suggest that plasminogen acquisition may be an important virulence determinant of GAS. The diversity of *emm* sequence types represented in this collection of isolates suggests that these relationships between plasminogen binding and invasive capacity do not depend on a small number of dominant clones.

The extent of direct plasminogen binding (4-81%) in this study is similar to that found in a previous study (9-69%) (36). Except for the conserved lysine residue in the A1 region, thought to be responsible for the majority of plasminogen binding (44) the plasminogen binding domain in PAM is highly divergent. The fact that PAM-negative isolates show only moderate reactivity with plasminogen despite the presence of the gene for SEN, another plasminogen binding protein, suggests that SEN may not play a major role in plasminogen sequestration.

Isolates from invasive disease cases bind significantly more plasminogen by the FSD pathway than isolates from uncomplicated infections. Plasminogen acquisition by the FSD pathway may therefore be a determinant for the invasive propensity. Plasmin activity owing to the FSD pathway is resistant to inhibition by plasma proteins, and has a half-life of over 4 h as opposed to 20 min for plasmin activity owing to direct plasminogen binding (38). The plasminogen-activation function of the complex also generates plasmin which can bind to surface receptors despite the presence of host physiological inhibitors (13). The significant enhancement of plasminogen binding has an absolute requirement for both fibrinogen and streptokinase, a result consistent with earlier reports (38, 39).

The difference in fibrinogen binding between PAM-positive and PAM-negative strains, correlation between fibrinogen and PAM-mediated plasminogen binding, and further enhancement of plasminogen binding by a combination of streptokinase and fibrinogen in PAM-positive isolates point to the occurrence of another receptor concomitant with PAM, or PAM itself may be endowed with dual receptor activity.

In contrast to PAM-positive isolates, neither the basal levels of plasminogen binding, nor the enhancement of the binding by the FSD pathway was correlated with fibrinogen binding to the surface of the PAM-negative isolates. This underscores the different mechanisms of plasminogen binding between PAM-positive and PAM-negative GAS strains.

The plasminogen system is only one of several factors implicated in the invasive process of GAS. The streptococcal pyrogenic exotoxin B of GAS possesses cysteine protease activity also capable of degrading host ECM (7, 21). The reason for lack of association between the plasminogen system and GAS invasive disease in the previous report (37) may simply reflect the analysis of the plasminogen system in isolation, and not as one of several factors in an overall proteolytic model. In this regard, overall proteolytic activity of human isolates has been associated with clinical signs of invasion (19, 35). Alternatively, these previous findings may simply be indicative of the complexity of the interaction of GAS and plasminogen in the invasive process. As Boyle and Lottenberg (1997) note, analysis of this complex system, involving at least four proteins, is particularly difficult as the bacterial components may be expressed only under certain environmental conditions (6).

In summary, we have demonstrated that GAS isolates from NT invasive disease cases belonging to a wide range of *emm* sequence types acquire more plasminogen than isolates from uncomplicated infections by a pathway requiring fibrinogen and streptokinase as cofactors. Among isolates containing the PAM gene, invasive isolates also bind more plasminogen directly. Subversion of the plasminogen system may be an important virulence determinant in GAS**,** and future work will focus on identifying the basis for higher plasminogen binding by invasive isolates within and between GAS genotypes.

Acknowledgments

We gratefully acknowledge the assistance of Associate Professor K. Russell of the Statistical Consulting Service, University of Wollongong, with statistical analysis of the data presented in this manuscript. We would also like to acknowledge the support of microbiology staff at Royal Darwin Hospital and Alice Springs Hospital in providing GAS isolates. We wish to thank Professor D. Bessen for providing and confirming some of our *emm* pattern and *emm* sequence data.

Fiona McKay is the recipient of a Biomedical Postgraduate Scholarship from the National Health and Medical Research Council, Australia. Martina Sanderson-Smith is the recipient of an Australian Research Council Postgraduate Award.

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Figure Legends

Figure 1. Translated DNA sequences of the A1 and A2 repeats of the 13 PAM-positive isolates aligned with the amino acid sequence of the prototype PAM gene. Isolates were considered PAM-positive if they produced a visible band on X-ray film with enhanced chemiluminescent detection (Supersignal® chemiluminescent substrate; Pierce, Rockford, IL) following Southern hybridisation, indicating the presence of a region with homology to the A1/A2 plasminogen-binding repeat region of PAM. Stars indicate residues identical to those of the PAM sequence, while dashes indicate gaps in the alignment. Percent amino acid sequence identity to PAM is also given. Despite low sequence homology of isolate NS696 with the prototype PAM amino acid sequence, the conservation of the lysine residue in the A1 region thought to be critical to plasminogen binding capacity of PAM, in addition to positive Southern hybridisation analysis, resulted in the PAM-positive designation of this isolate.

Figure 2. ^{125}I-plasminogen binding to PAM-positive (●) and PAM-negative (○) group A streptococcal isolates from invasive and uncomplicated infections. A. Direct binding of ^{125}I-plasminogen. B. Binding of ^{125}I-plasminogen in the presence of molar excesses of streptokinase and fibrinogen. All estimates were determined in triplicate and data are presented as mean. Standard error of the mean was 0-6% of input ^{125}I-plasminogen.

Figure 3. Binding of ^{125}I-plasminogen to group A streptococcal isolate NS1133 under different conditions. ^{125}I-plasminogen binding was measured in the presence of the lysine analogue, eACA (Plg + eACA); in the presence of no other added reagents (Plg only); in

the presence of streptokinase (Plg + Ska); in the presence of fibrinogen (Plg + Fg); and in the presence of streptokinase and fibrinogen (Plg + Ska + Fg). All reagents were added in molar excess relative to ^{125}I-plasminogen. All estimates were determined in triplicate and data are presented as mean $\pm$ SEM. Significant differences, as determined by unpaired *t* test, from the binding of ^{125}I-plasminogen alone (Plg only) are marked (*).

Figure 4. Comparison of ^{125}I-plasminogen binding to PAM-positive (●) and PAM-negative (○) group A streptococcal isolates in the presence of streptokinase and fibrinogen with ability of the isolates to bind ^{125}I-fibrinogen or ^{125}I-plasminogen directly. All estimates were determined in triplicate and data are presented as mean. Correlation curves are shown for relationships with significant correlation. A. Comparison with binding of ^{125}I-fibrinogen by group A streptococcal isolates with a correlation curve fitted to PAM-positive isolates ($r = 0.775$, $p \leq 0.002$). B. Comparison with direct binding of ^{125}I-plasminogen by group A streptococcal isolates with a correlation curve fitted to PAM-positive isolates ($r = 0.902$, $p \leq 0.0001$).

Table 1. Primers designed for DNA methods

Primer	DNA Sequence	Use
SENF1	5'-GGTATGGATGAAAACGACTGGGATG-3'	PCR amplification and
SENR1	5'-TGTCGTGACCAACCTAGTCAGCCTG-3'	sequencing of SEN gene
PAMF1	5'-ATAAGCAAGAACATCTTGACGG-3'	
PAMR1	5'-CTGTTAATTTCTTGCTTTC-3'	Sequencing of PAM
PAMF2	5'-AAAGGGCTTAAGACTGATTTAC-3'	genes[a]
PAMR2	5'-GACCAGCTAATTTGCTGTTTGC-3'	
PAMR3	5'-CTTCTCAACATCATCTTTAAGG-3'	

[a]PAM gene sequence was determined using M13LacZ forward and universal reverse primers (Perkin Elmer, Boston, MA), primers N1 and N2 flanking the plasminogen-binding site of PAM (5) and the primers listed above, designed to anneal to various sites within the PAM gene.

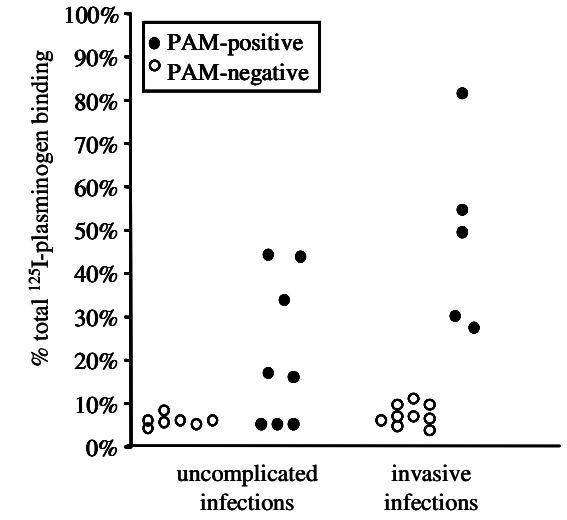
Table 2. Characteristics of the 29 NT group A streptococcal isolates

Isolate	Clinical origin	Invasive category[a]	PAM status[b]	^{125}I-plg binding (±SEM;%)	Enhanced ^{125}I-plg binding[c] (±SEM;%)	*Vir* type[d]	*emm* sequence type[e]	*emm* pattern[f]
NS192	renal transplant, septic (blood)	inv	-	6(±0)%	29(±1)%	3.2	100	E
NS210	diabetic ulcer with fever	inv	-	7(±0)%	29(±2)%	34	22	E
NS414	wound, cellulitis	inv	-	4(±0)%	27(±2)%	3.3	11	E
NS452	cellulitis/ wound	inv	-	7(±0)%	57(±1)%	45	25	E
NS501	blood	inv	-	10(±1)%	63(±6)%	61	14	ABC
NS730	necrotising fasciitis, pus from left hip	inv	-	11(±0)%	55(±1)%	2.2	90	E
NS733	necrotising fasciitis, wrist aspirate	inv	-	5(±1)%	29(±0)%	2.2	90	E
NS931	necrotising fasciitis, blood	inv	-	6(±0)%	52(±3)%	57	69	D
NS179	pustules on foot, bacteremia	inv	-	9(±1)%	57(±1)%	7.2	9.1	E
NS13	blood	inv	+	81(±2)%	89(±1)%	24	53	D
NS88.2	blood	inv	+	54(±1)%	60(±1)%	17.4	98.1	D
NS223	infected CV line (leg), blood	inv	+	27(±0)%	55(±2)%	4	91	D
NS455	blood	inv	+	30(±1)%	54(±1)%	29.1	52	D
NS1133	blood	inv	+	50(±1)%	81(±1)%	17.1	101	D
NS14	post-operative wound	uncomp	-	4(±0)%	27(±0)%	96	102	E
NS236	sore throat	uncomp	-	6(±0)%	27(±0)%	111	77	E
NS244	wound	uncomp	-	8(±0)%	59(±1)%	14.1	hybrid emm4/st 11014	ABC[g]
NS297	skin sore	uncomp	-	6(±1)%	50(±0)%	3.1	44/61	E
NS474	wound	uncomp	-	5(±0)%	24(±0)%	22	58	ABC[g]
NS488	sinusitis/ persistent pharyngeal pus well	uncomp	-	6(±0)%	5(±0)%	52	12	ABC
NS836	wound	uncomp	-	5(±0)%	32(±1)%	46	ck249	D
NS10	throat	uncomp	+	44(±2)%	54(±0)%	24	53	D
NS32	wound	uncomp	+	34(±0)%	58(±1)%	29.2	101	D
NS50.1	wound	uncomp	+	17(±1)%	46(±3)%	12.1	108	D
NS53	febrile/unwell	uncomp	+	5(±0)%	35(±1)%	29.1	71	D
NS59	wound	uncomp	+	44(±1)%	61(±1)%	24	53	D
NS253	wound	uncomp	+	15(±1)%	31(±6)%	29.1	52	D
NS265	wound	uncomp	+	5(±0)%	21(±1)%	11	56	D
NS696	throat swab, pharyngitis	uncomp	+	5(±0)%	7(±1)%	78	1	ABC

[a] invasive (inv) or uncomplicated (uncomp) GAS infection classification according to sterile or nonsterile tissue site of infection, respectively.
[b] positive (+) or negative (-) plasminogen-binding group A streptococcal M-like protein (PAM) status according to hybridisation of M or M-like gene from GAS isolate with an oligonucleotide probe homologous to the A1/A2 repeat of the PAM gene by Southern analysis.
[c] Binding of ^{125}I-plasminogen to GAS in the presence of excess streptokinase and fibrinogen.
[d] *Vir* type was determined as previously described (20).
[e] emm sequence type was determined using a protocol modified from the *Streptococcus pyogenes emm* sequence database (4).
[f] emm pattern was determined as previously described (6, 24) except that annealing temperatures varied between 56°C and 62°C depending on the strain.
[g] isolates NS244 and NS474 gave an unusual *emm* pattern, in that PCR product resulted only from reaction with primer set 2, rather than sets 1, 2, and 3; or set 1 only as for traditional *emm* pattern ABC.

	A1	A2	
PAM	DAELQRLKNERH---E	EAELERLKSERHD	Percent PAM Homology
NS10	**********	**********	100%
NS13	**********	**********	100%
NS59	**********	**********	100%
NS50.1	**********	**********E	96.5%
NS1133	A***E**********D	HD******N****	79%
NS32	A***E**********D	HD******N*G**	76%
NS53	EVA*E*******VHD*	*V******N****	69%
NS253	EVA*E*******VHD*	*V******N****	69%
NS455	EVA*E*******VHD*	*V******N****	69%
NS223	EV**E*******DHD*	****N**RE****	69%
NS265	EVA*E*******VHD*	********N***Y	65%
NS88.2	ER**EDL*----***-	D***K**NE****	52%
NS696	WDRQRLE*ELEE**KK	EALAIDQA*RDHY	21%

Figure 1


100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% total 125I-plasminogen binding
PAM-positive
PAM-negative
uncomplicated infections
invasive infections

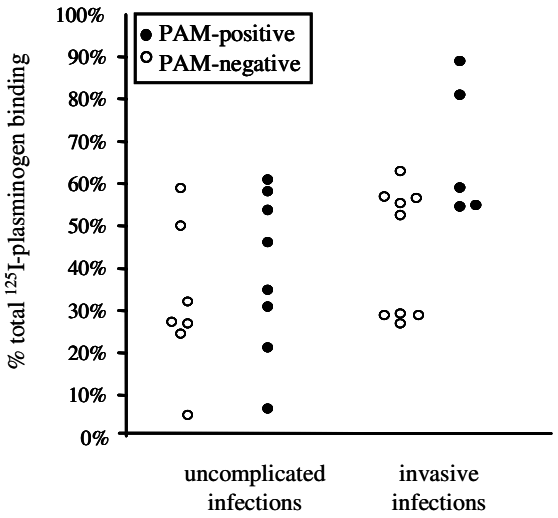

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% total 125I-plasminogen binding
PAM-positive
PAM-negative
uncomplicated infections
invasive infections

Figure 2

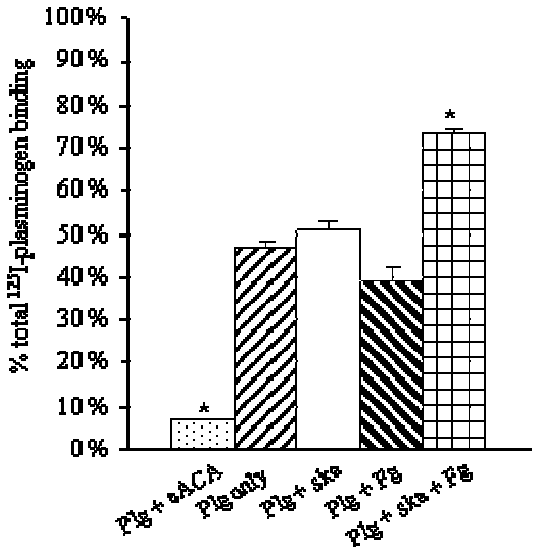

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
% total [125]I-plasminogen binding
*
*
Plg+ εACA
Plg only
Plg+ Ska
Plg+ Pg
Plg+ Ska+ Pg

Figure 3

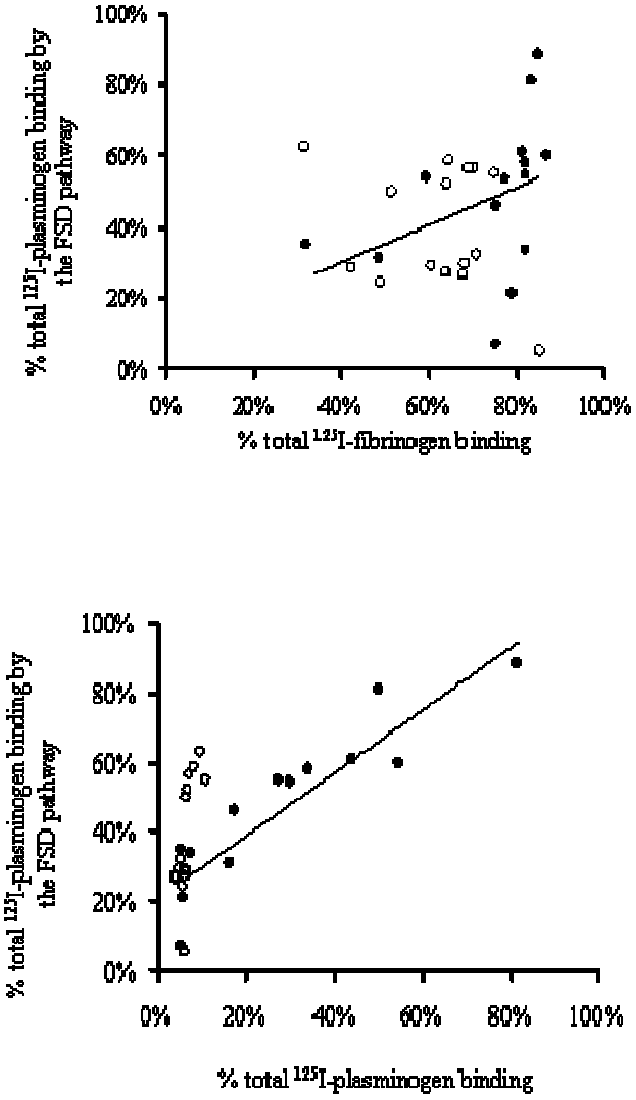

100%
80%
60%
40%
20%
0%
0%
20%
40%
60%
80%
100%
% total 125I-plasminogen binding by the FSD pathway
% total 125I-fibrinogen binding
% total 125I-plasminogen binding by the FSD pathway
% total 125I-plasminogen binding

Figure 4